CURRENTA

CURRENTA GmbH & Co. OHG
Analytik
CHEMPARK, Gebäude K 46
51368 Leverkusen

1. Amendment to GLP-Final Report

Study Title: Alga, Growth Inhibition Test with PES Vorstufe 2342

Study number: 2010/0087/10

Test item: PES Vorstufe 2342

Date: 2011-05-25

CAS number: ---

Correction of the GLP-final report: ☒ **Addition to the GLP-final report:** ☐

Reason: The structural formula on page 12 is incorrect.

Correction / Addition: The structural formula is deleted without replacement.

Study director 2011-05-25 A. Neuhahn

Date / Signature

Enclosure: QS-statement for the amendment

CURRENTA GmbH & Co. OHG
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Statement of the Quality Assurance on the Final Report

Key of the GLP-study: 2010/0087/10

Test substance: PES Vorstufe 2342

Title of the GLP-study: Alga, growth inhibition test

This GLP-study was inspected by the quality assurance.
The dates of inspections and the dates of reports to the management and the study director are:

phase	date of inspection	date of report
final report review / 1. Amendement	30.05.2011	30.05.2011

The results shown in the final report on this study were inspected on the basis of the current SOPs/analytical methods. It is confirmed, that the report results to the best of our knowledge reflect the raw data of the study.

Quality assurance:

2011-05-30
Date

A. Seus
Signature

Study Title

Alga, Growth Inhibition Test with

PES Vorstufe 2342

Data Requirements / Test Guidelines

EU method C.3 (2009)

OECD TG 201 (2006)

Author:

Astrid Neuhahn

Study completion date:

2011-05-04

Sponsor:

Bayer MaterialScience AG

BMS-IO-ST-PSRA-PRA

51368 Leverkusen

Germany

Testing facility:

CURRENTA GmbH & Co. OHG

Analytik

51368 Leverkusen

Germany

Monitor:

Dr. Ralf Werner

BMS-IO-ST-PSRA-PRA

51368 Leverkusen

Germany

Laboratory Project Identification

Study No. 2010/0087/10

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CONTENTS

1. GLP DECLARATION

This study was conducted in compliance with the OECD principles of Good Laboratory Practice (1999) and with the Principles of Good Laboratory Practice according to Annex I, German Chemical Law (2008).

Date / Signature

Study Director 2011-05-04 A. Neuhahn
(Neuhahn / ~~Dr. Richter~~)

2. ARCHIVING

The original report, the study plan, and all raw data pertaining to this study are stored in the "GLP Archive, CURRENTA GmbH & Co. OHG, Analytik, CHEMPARK, Building Q 18, 51368 Leverkusen". A sample of the test item is stored in "GLP-Sample Store, CURRENTA GmbH & Co. OHG, Analytik, CHEMPARK, Building DA1, 41538 Dormagen".

This page is intentionally left blank for the purpose of submitting administrative information that is required by regulations promulgated by various countries.

3. QUALITY ASSURANCE STATEMENT

This report was audited by the Quality Assurance Unit CURRENTA Analytik, Quality Management at CURRENTA GmbH & Co. OHG and this statement confirms that the final report reflects the raw data.

The dates of Quality Assurance inspections and audits are given below.

Audits	Dates of QAU inspections	Dates of reports
study plan review	2011-03-17	2011-03-17
study plan review	2011-03-22	2011-03-22
process based inspection	2010-12-07 2010/0135/01	2010-12-07
final report review (draft)	2011-05-04	2011-05-04
final report review	2011-05-05	2011-05-05

Date / Signature

2011-05-05 A. Senic

(Senic/ ~~Dr. Dörzbach-Lange/ Dr. Neupert~~)

4. STUDY TIME TABLE

Study initiation date:	2011-03-17
Study completion date:	2011-05-04
Start of experimental phase:	2011-03-28
End of experimental phase:	2011-04-28

5. GLP CERTIFICATE

Ministerium für Umwelt und Naturschutz, Landwirtschaft und Verbraucherschutz
des Landes Nordrhein-Westfalen

Postanschrift: 40190 Düsseldorf

Aktenzeichen: VI-3- 31.11.65.05

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Bayer Industry Services GmbH & Co OHG
Prüfeinrichtung BIS-SUA-Analytics
D-51368 Leverkusen

(unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien (gemäß ChemVwV-GLP Nr. 5.3/OECD guidance)	Areas of Expertise (according ChemVwV-GLP Nr. 5.3/OECD guidance)
Kategorie 1	category 1
Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	physical-chemical testing
Kategorie 4	category 4
Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5	category 5
Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	studies on behaviour in water, soil and air; bioaccumulation
Kategorie 8	category 8
Analytische Prüfungen an biologischen Materialien	analytical and clinical chemistry testing

Datum der Inspektion (Tag.Monat.Jahr)	Date of Inspection (day.month.year)
14. bis 16. September und 26. bis 28. Oktober 2005	on 14 until 16 September and on 26 until 28 October 2005
Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.	The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.	Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Düsseldorf, den 11. Januar 2006
Im Auftrag

(Prof. Dr. David)

Dienstsiegel/official-seal

Please note: Effective January 1st, 2008 the company name Bayer Industry Services GmbH & Co. OHG was changed to CURRENTA GmbH & Co. OHG

6. SUMMARY

A study was performed to assess the adverse effects of PES Vorstufe 2342 on the growth rate (= rate of increase in cell density with time) and the yield (= biomass at time t minus initial biomass) of the planktonic freshwater algal species *Desmodesmus subspicatus* (former name: *Scenedesmus subspicatus*) over several generations.

The study was conducted in accordance with Commission Regulation (EC) No 761/2009 amending Regulation No 440/2008, Method C.3 'Freshwater Alga and Cyanobacteria, Growth inhibition test' (2009) which is equivalent to OECD Guideline for Testing of Chemicals No. 201 (2006).

Exponentially growing algal cells were exposed for a period of 72 hours to a limit test concentration of nominally 100 mg/L of PES Vorstufe 2342 dissolved in dilution water. Auxiliaries used to prepare the test media were an ultra turrax, a magnetic stirrer and an aseptic filter.

The cell densities were measured at 24 hour intervals. Inhibition of the algal population was measured as reduction in growth rate (index r), relative to control cultures grown under identical conditions. The following values were determined:

Results [mg/L]:

E_rL 50* (0-72 h): >100

E_rL 10* (0-72 h): >100

NOEL [r] ($t_{\alpha\ 0.05}$): ≥100

LOEL [r] ($t_{\alpha\ 0.05}$): >100

No toxic effects against algae were observed at a limit test concentration of 100 mg/L.

* Reduction of growth rate (E_rLx, NOEL [r]) is the preferred endpoint according to OECD 201 and for regulatory purposes in the EU. Results relating to yield (E_yLx, NOEL [y]) were calculated to fulfil regulatory requirements in some countries (but not in the EU) and are given in the results section of this report.

PES Vorstufe 2342 is insoluble or poorly soluble in water. Therefore a suitable selective and sensitive chromatographic method for the determination of the test item in aqueous solutions could not be established.

The results are expressed in terms of Effective Loadings (EL). As the test item is a multi constituent and no information about the correlation between molecular weight and the structural formula of the test item are available, a Water Accommodated Fraction (WAF) was used to test effects at a limit concentration of 100 mg/L, and no specific analysis was performed. With the sponsor's agreement, the content of the test item during the exposure period was verified by DOC determination.

7. EXPERIMENTAL PROCEDURE

This report contains a description of the methods used and the results obtained during a study to investigate adverse effects of PES Vorstufe 2342 on the growth rate (and yield) of a population of the planktonic freshwater algal species *Desmodesmus subspicatus* (former name: *Scenedesmus subspicatus*).

The study was conducted in accordance with Commission Regulation (EC) No 761/2009 amending Regulation No 440/2008, Method C.3 ‘Freshwater Alga and Cyanobacteria, Growth inhibition test’ (2009) which is equivalent to OECD Guideline for Testing of Chemicals No. 201 'Alga, Growth Inhibition Test' (2006).

The purpose of this method was to determine those concentrations which caused a 10 % and a 50 % adverse effect (= EC 10, EC 50) or, if conducted as a limit test, to determine the adverse effects at a maximum test concentration of 100 mg/L or at the limit of water solubility. Effect data are expressed on the basis of growth rate [r] (and yield [y]).

A range finding (non-GLP) test preceded the main test and provided information about the range of concentrations which were used in the main test. The following nominal concentrations of the test item were tested in the range finding test: 1, 10 and 100 mg/L.

In the main test, the algae were exposed to the test item added to dilution water at a limit Effective Loading of nominally 100 mg/L for a period of 72 hours. At this concentration no inhibition of algal growth rate and yield was observed at the end of the 72 hour study period. Cell densities were recorded at 24 hour intervals.

All calculations were carried out using the statistics programme ToxRatPro Version 2.10 (released 2010-02-20). For the calculations all algae counts were divided by a factor of 10000.

During the test a temperature range of 21 - 24°C was maintained in the test vessels.
The pH was measured at the beginning of the test and after 72 hours of exposure.

The maintenance of the test item concentration was proved by analytical measurements. In order to avoid an impairment of the test system, an additional replicate was used for analysis and pH measurement at the beginning of the test. Chemical analysis and pH measurement at the end of the test were performed using replicate I of the test concentrations and the control vessels.

In order to check whether or not significant amounts of the test item were incorporated into the algal biomass during the test period, a test flask at the Effective Loading without algae was run in parallel to the test concentration.

The following validity criteria of the test were met:

The cell density in the control cultures increased by a factor of at least 16 within 72 hours.

The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3, for 72-hour tests) in the control cultures did not exceed 35 %.

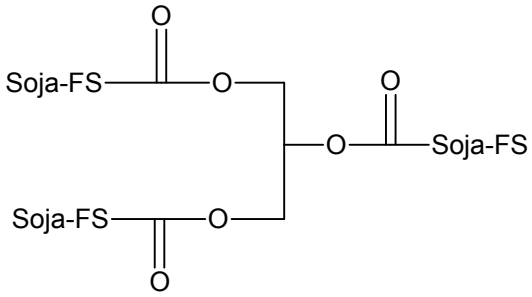
The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7 %.

8. MATERIALS AND METHODS

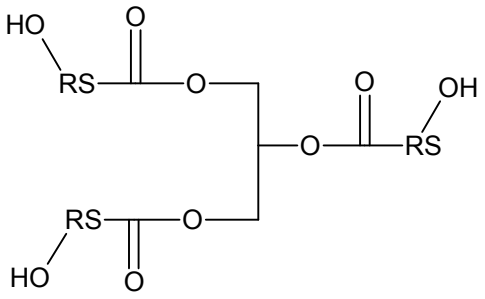
8.1 Sample description

Test item	:	PES Vorstufe 2342
Chemical name	:	Castor Oil, reaction product with Soybean Oil
CAS name	:	--
CAS number	:	--
EC/NLP number	:	919-697-6
Sample provided by	:	Bayer MaterialScience
Empirical formula	:	--
Molecular mass	:	-- g/mol
Structural formula	:	

Reaction product of castor oil
and soy bean oil (Transesterification)



Soja-FS = Soybean oil fatty acid



RS = Castor oil fatty acid

Batch number	:	LB06603520
Charge	:	--
Sample number	:	1199
Date of receipt	:	2010-04-27
Expiry date	:	2011-09-11
Purity	:	100 % (according to data of the sponsor)
Water solubility	:	0.0058 g/l
Density	:	0.95 g/cm^3 at 20°C
Vapour pressure	:	ca. 4 hPa at 20°C
Stability of test concentration/s during exposure	:	Examined by chemical analysis (DOC) at 0 and 72 hours.

8.2 Test species

Name	:	*Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*) Strain No. 86.81 SAG
Source	:	Strain of the test species obtained from 'The Collection of Algal Cultures' of the Institute of Plant Physiology at the University of Göttingen (Germany).
Maintenance and Acclimatisation	:	Exponentially growing stock cultures were maintained in the test facility under constant temperature conditions (21-24 °C with a maximum fluctuation of +/- 2 °C) at a light intensity in the range 60 to 120 µE x m^{-2} x s^{-1} (measured in the range 400 to 700 nm using a spherical quantum flux meter). The growth medium (according to BRINGMANN & KÜHN (1977) was renewed once a week. Cell density measurements were made using a microcell counter, Sysmex F300, Digitana.
Preparation of pre cultures	:	Pre cultures were set up three days before the start of a test. They were grown under identical exposure conditions as the stock cultures, except from the use of a different growth medium (annex 1).
Test cultures	:	The algal inocula for the test were taken from an exponentially growing pre culture and were mixed with the growth medium (annex 1) to make up to a final cell density of about 5000 cells per millilitre in the test medium.

8.3 Growth medium and dilution water

Growth medium (OECD medium of OECD TG 201, annex 1) was used for the growth of the algae in the pre cultures and the preparation of stock and test solutions of the test item.

8.4 Apparatus

Analytical balance

pH meter

Shaking incubator

Microcellcounter

Various glass materials: Erlenmeyer flasks, volumetric flasks, beakers, pipettes etc.

8.5 Pre-treatment of test item and preparation of test item concentrations

To produce the only test item concentration 100.4 mg of the test item were added to 1 litre of dilution water and treated for one minute at 8000 rpm with an ultra turrax and afterwards stirred for 24 h on a magnetic stirrer. Undissolved particles of the test item were removed by filtration using an aseptic filter, Sartobran 150, with a pore size of 0.45+0.2 µm. The pH was measured to be 7.5.

100 mL of the solution were taken and 0.555 mL of the algal inoculum was added to each replicate resulting in a final cell density of 5000 cells/mL. For each test item concentration and the control 6 replicates were prepared. All flasks were sealed with cotton stoppers.

8.6 Exposure conditions

Test vessels	:	300 mL Erlenmeyer flasks with cotton stoppers test volume: 100 mL
Culturing apparatus	:	Light chamber in which a temperature in the range 21 °C to 24 °C was maintained at +/- 2 °C, and continuous uniform illumination was provided in the spectral range 400 to 700 nm. Temperature was measured and recorded daily in a water filled flask which was incubated under the same conditions as the test flasks.

Light intensity	:	A light intensity ranging from 60 to 120 µE x m^{-2} x s^{-1}, or an equivalent range of 4000 to 8000 lux, was measured. The light intensity was checked before the start of the study.
Cell density measurements	:	Cell densities were measured in a microcell counter (Sysmex F300, Digitana) by taking small aliquots from each test flask, which were not replaced.
Experimental design	:	1 test concentration plus 1 control 6 replicates per concentration, 6 replicates per control Initial cell density in the test cultures approximately 5000 cells per millilitre. Additionally highest test concentration without algae.
Test item concentration/s	:	100 mg/L
Method of administration	:	direct weighing
Duration of exposure	:	72 hours
Criteria of effects	:	The criteria of adverse effects used in this study were the item-induced inhibition of yield [y] and growth rate [r] of the algal population.

8.7 Chemical analysis

PES Vorstufe 2342 is insoluble or poorly soluble in water. Therefore a suitable selective and sensitive chromatographic method for the determination of the test item in aqueous solutions could not be established.

An analytical confirmation of the test concentration by means of a chromatographic method could not be performed. With the sponsor's agreement, the content of the test item during the exposure period was verified by DOC determinations.

Analytical Standards

Analytical Standard for Determination of Organic Carbon

Potassium hydrogen phthalate, dried at 105°C for 1 hour, purity > 99.9 %
Potassium hydrogen phthalate (nominal value: 2.125 g) was dissolved in water and made up to the mark in a 1000 mL volumetric flask to prepare a stock solution of 1000 mg Carbon per litre. Defined volumes of the stock solution were diluted with water to obtain standard solutions in the range of 5 to 300 mg/L.

Analytical Standard for Determination of Inorganic Carbon

Sodium carbonate, dried at 285°C for 1 hour, purity > 99.9 % Sodium hydrogen carbonate, dried for 2 hours over silica gel, purity > 99.9 %
Sodium carbonate (nominal value: 4.415 g) was dissolved in about 500 mL water. Sodium hydrogen carbonate (nominal value: 3.500 g) was added and made up to the mark in a 1000 mL volumetric flask to prepare a stock solution of 1000 mg Carbon per litre. Defined volumes of the stock solution were diluted with water to obtain standard solutions in the range of 15 to 150 mg/L.

Analytical Procedure

Principle

Total Carbon (TC) in water was oxidized to carbon dioxide by combustion. Inorganic Carbon (IC) was measured separately by acidification and purging. Total Organic Carbon (TOC) was calculated by the following equation:

$$TOC = TC - IC$$

As the bioavailable fraction of organic test items is more appropriately reflected by the Dissolved Organic Carbon (DOC), all biological test solutions were initially filtered through a membrane filter of a pore size of 0.45 µm before any further treatment was performed. In case of low DOC values (< 10 mg/L), DOC was measured after removing inorganic carbon by acidification and purging of carbon dioxide. In this case, DOC value was identical with TC.
Carbon dioxide was determined directly by infrared spectrometry.

Calibration

Linear calibration curves were established by analysing organic standard solutions and inorganic carbon solutions of at least three adequate concentrations. Typically, several calibration curves were used in order to cover the whole concentration range needed.

Limit of quantitation

2 mg/L DOC.

Analysis of samples

The biological test solutions were routinely measured on the day of sampling. If this was exceptionally not possible, the samples were stored in a refrigerator at 4 °C until the analysis was carried out. The biological test solutions were analysed in the same way as the calibration samples.

Evaluation of results

Injected samples were quantified by peak areas with reference to the respective calibration curve. The latter was obtained by correlation of peak area of the standard solutions to their corresponding concentration in mg/L. The correlation was performed using a linear function:

$$y = m \cdot x + b$$

y	= peak area of injected sample (counts)
x	= DOC of injected sample (mg carbon per litre)
m	= constant factor, slope of calibration curve
b	= intercept, point of intersection between calibration curve and y-axis

Factor 'Molecular weight / Organic C content': ---

Sampling schedule:

Control	:	at 72 hours
Test concentration	:	at 0 and 72 hours

8.8 Expression of biological results

Cell density measurements, yield and growth rates in the test and control cultures were tabulated according to the concentration of the test item and the time of measurement.

Growth curves were plotted for the test concentration and the control and are presented in **figure 1**.

The percentage inhibition of both, yield [y] and growth rate [r], was calculated for the test concentration using equations **[1]** and **[3]** in **annex 2**.

The growth rate [r] was calculated for each test concentration using equation **[2]** in **annex 2**.

A **limit test** was performed, in order to demonstrate that the EL 50 was greater than this concentration.

8.9 Applied SOPs and methods

00319 V.1	Algal growth inhibition test
2011-0615201-07D	DOC determination

Deviations: none

9. RESULTS

Cell number in Desmodesmus subspicatus as Dependent on Concentration and Time

(upon program request, values of cell number were divided by a factor of 10000)

Tab. 1: Cell number in Desmodesmus subspicatus as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg/L]	Control	100
0 h	0.500	0.500
	0.500	0.500
	0.500	0.500
	0.500	0.500
	0.500	0.500
	0.500	0.500
Mean:	0.500	0.500
Std.Dev.:	0.0000	0.0000
n:	6	6
CV:	0.0	0.0
24 h	2.000	2.000
	2.000	2.000
	2.000	2.000
	2.000	3.000
	2.000	2.000
	2.000	2.000
Mean:	2.000	2.167
Std.Dev.:	0.0000	0.4082
n:	6	6
CV:	0.0	18.8
48 h	8.000	8.000
	8.000	7.000
	8.000	7.000
	10.000	8.000
	11.000	8.000
	10.000	8.000
Mean:	9.167	7.667
Std.Dev.:	1.3292	0.5164
n:	6	6
CV:	14.5	6.7

Tab. 1 (continued): Cell number in Desmodesmus subspicatus as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

72 h	41.000	62.000
	47.000	59.000
	49.000	57.000
	45.000	61.000
	45.000	64.000
	48.000	60.000
Mean:	45.833	60.500
Std.Dev.:	2.8577	2.4290
n:	6	6
CV:	6.2	4.0

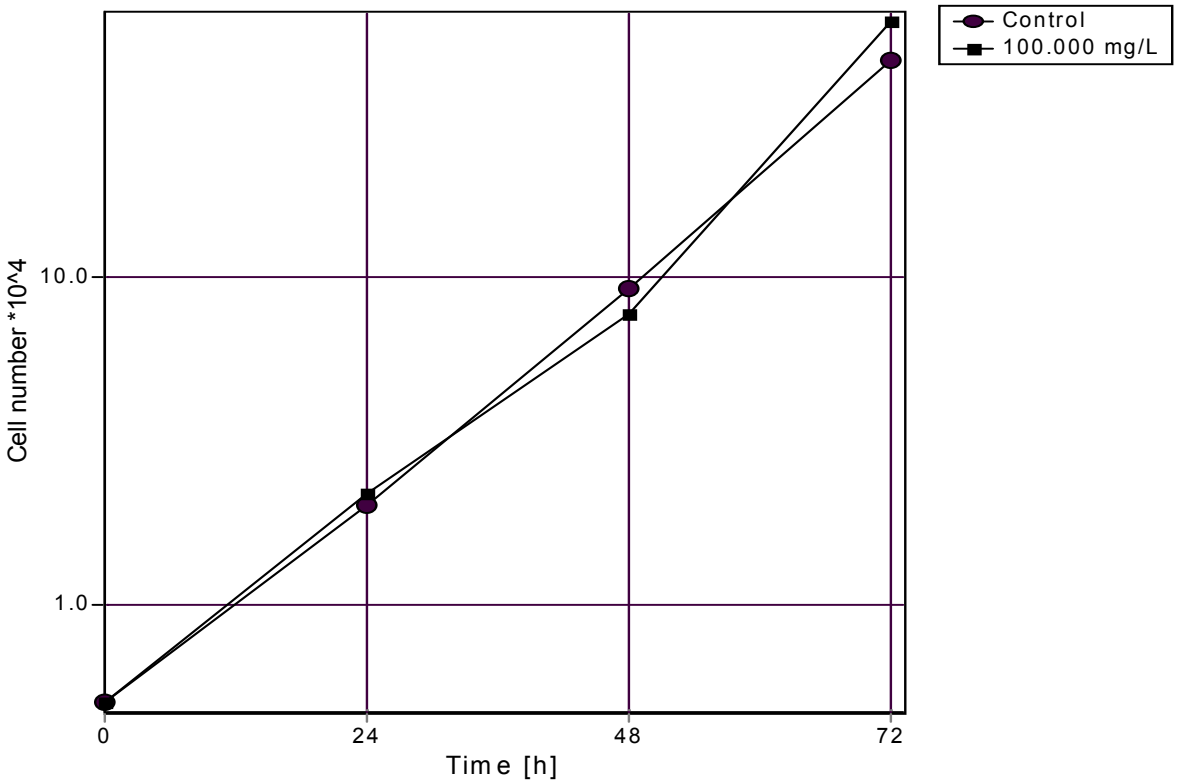


Fig. 1: Cell number in Desmodesmus subspicatus as dependent on test item concentration and time.

Yield of Desmodesmus subspicatus cells as Dependent on Concentration and Time

Tab. 2: Yield of Desmodesmus subspicatus cells as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg/L]	Control	100
0 h	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
Mean:	0.000	0.000
Std.Dev.:	0.0000	0.0000
n:	6	6
CV:		
24 h	1.500	1.500
	1.500	1.500
	1.500	1.500
	1.500	2.500
	1.500	1.500
	1.500	1.500
Mean:	1.500	1.667
Std.Dev.:	0.0000	0.4082
n:	6	6
CV:	0.0	24.5
48 h	7.500	7.500
	7.500	6.500
	7.500	6.500
	9.500	7.500
	10.500	7.500
	9.500	7.500
Mean:	8.667	7.167
Std.Dev.:	1.3292	0.5164
n:	6	6
CV:	15.3	7.2

Tab. 2: Yield of Desmodesmus subspicatus cells as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

72 h	40.500	61.500
	46.500	58.500
	48.500	56.500
	44.500	60.500
	44.500	63.500
	47.500	59.500
Mean:	45.333	60.000
Std.Dev.:	2.8577	2.4290
n:	6	6
CV:	6.3	4.0

Yield of Desmodesmus subspicatus

Tab. 3: %Inhibition caused by the test item after 72 h.

Treatm.[mg/L]	Mean	Std. Dev.	n	%Decrease
Control	45.333	2.8577	6	0.0
100	60.000	2.4290	6	-32.4

Effects on Yield

Tab. 4: Yield (Y) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0-24 h		0-48 h		0-72 h	
[mg/L]	Y	%I	Y	%I	Y	%I
Control	1.500	0.0	8.667	0.0	45.333	0.0
100	1.667	-11.1	7.167	17.3	60.000	-32.4

Threshold Concentrations (NOEC) for Yield at 72 h

Statistical Characteristics of the Sample

Tab. 5: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	45.333	45.500	40.500	48.500	6	2.8577	6.3	1.1667	2.6	42.326	48.341
100	60.000	60.000	56.500	63.500	6	2.4290	4.0	0.9916	1.7	57.444	62.556

Shapiro-Wilk´s Test on Normal Distribution

Tab. 6: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [mg/L]	Mean	s	n
Control	45.333	2.8577	6
100	60.000	2.4290	6

Results:

Number of residues = 11; Shapiro-Wilk´s W = 0.956; p(W) = 0.675; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s test is chosen for variance homogeneity testing.

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 7: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	10.7037	1	10.7037	0.192	0.671
Residuals	558.5926	10	55.8593		
Total	569.2963	11			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity !

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

STUDENT-t test for Homogeneous Variances

Tab. 8: Pair-wise comparison of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: $\mu 1 = \mu 2$; the differences are significant in case p(i) <= Alpha ; p(F): probability of F computed by a F-test (Ho: var1 = var2 (homogeneity); p(F) > 0.05 is the criterion of variance homogeneity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg/L]	Mean	s^2	df	%MDD	t	p(t)	Sign.	p(F)
Control	45.333	7.033						
100	60.000	7.033	10	-6.120	9.58	1.000	-	0.365

+: significant; -: non-significant; n.d.: not determined

There is no statistically significant difference between Control and 100 mg/L.

Mean specific growth rate in Desmodesmus subspicatus as Dependent on Concentration and Time

Tab. 9: Mean specific growth rate in Desmodesmus subspicatus as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg/L]	Control	100
0 h	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
	0.000	0.000
Mean:	0.000	0.000
Std.Dev.:	0.0000	0.0000
n:	6	6
CV:		
24 h	1.386	1.386
	1.386	1.386
	1.386	1.386
	1.386	1.792
	1.386	1.386
	1.386	1.386
Mean:	1.386	1.454
Std.Dev.:	0.0000	0.1655
n:	6	6
CV:	0.0	11.4
48 h	1.386	1.386
	1.386	1.320
	1.386	1.320
	1.498	1.386
	1.546	1.386
	1.498	1.386
Mean:	1.450	1.364
Std.Dev.:	0.0719	0.0345
n:	6	6
CV:	5.0	2.5

Tab. 9 (continued): Mean specific growth rate in Desmodesmus subspicatus as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

72 h	1.469	1.607
	1.514	1.590
	1.528	1.579
	1.500	1.601
	1.500	1.617
	1.521	1.596
Mean:	1.505	1.598
Std.Dev.:	0.0212	0.0134
n:	6	6
CV:	1.4	0.8

Growth Rate of Desmodesmus subspicatus

Tab. 10: %Inhibition caused by the test item after 72 h.

Treatm.[mg/L]	Mean	Std. Dev.	n	%Inhibition
Control	1.505	0.0212	6	0.0
100	1.598	0.0134	6	-6.2

Effects on Growth Rate

Tab. 11: Growth rate (G) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0-24 h		0-48 h		0-72 h	
[mg/L]	G	%I	G	%I	G	%I
Control	1.386	0.0	1.450	0.0	1.505	0.0
100	1.454	-4.9	1.364	5.9	1.598	-6.2

Threshold Concentrations (NOEC) for Growth rate at 72 h

Statistical Characteristics of the Sample

Tab. 12: Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	1.505	1.507	1.469	1.528	6	0.0212	1.4	0.0087	0.6	1.483	1.528
100	1.598	1.599	1.579	1.617	6	0.0134	0.8	0.0055	0.3	1.584	1.612

Shapiro-Wilk´s Test on Normal Distribution

Tab. 13: Shapiro-Wilk´s Test on Normal Distribution; Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic. In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [mg/L]	Mean	s	n
Control	1.505	0.0212	6
100	1.598	0.0134	6

Results:

Number of residues = 11; Shapiro-Wilk´s W = 0.947; p(W) = 0.632; p(W) is greater than the selected significance level of 0.05; therefore, treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0.05).

Levene´s test is chosen for variance homogeneity testing.

Levene´s Test on Variance Homogeneity (with Residuals)

Tab. 14: Levene´s Test on Variance Homogeneity (with Residuals): Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability

Source	SS	df	MSS	F	p(F)
Treatment	0.0000	1	0.0000	1.066	0.326
Residuals	0.0000	10	0.0000		
Total	0.0000	11			

Based on the pre-selected significance level of 0.05, the Levene test indicates variance homogeneity !

Variance homogeneity check was passed.
Normal distribution and variance homogeneity requirements are fulfilled.
A parametric multiple test is advisable.

STUDENT-t test for Homogeneous Variances

Tab. 15: Pair-wise comparison of treatments with "Control" by the t test procedure. Significance was Alpha = 0.05, one-sided smaller; Mean: arithmetic mean; n: sample size; s^2: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: μ1 = μ2; the differences are significant in case p(i) <= Alpha ; p(F): probability of F computed by a F-test (Ho: var1 = var2 (homogeneity); p(F) > 0.05 is the criterion of variance homogeneity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg/L]	Mean	s^2	df	%MDD	t	p(t)	Sign.	p(F)
Control	1.505	0.000						
100	1.598	0.000	10	-1.234	9.06	1.000	-	0.167

+: significant; -: non-significant; n.d.: not determined

There is no statistically significant difference between Control and 100 mg/L.

Effects on Section-by-section Growth Rate

Tab. 16: Section-by-section growth rate (SG) and its inhibition relative to control (%I) as computed from the raw data for test intervals selected.

Treatment	0 - 24 h		24 - 48 h		48 - 72 h	
[mg/L]	SG	%I	SG	%I	SG	%I
Control	1.386	0.0	1.514	0.0	1.616	0.0
100	1.454	-4.9	1.274	15.8	2.067	-27.9

Table 17, Analysis

Test item concentration [mg/L]	DOC values [mg/L]	
	0 h	72 h
Control	n.d.	3.00
100	3.31	4.87
100 without algae	3.68	<2

Comments: n.d. not determined

Table 18, pH values at the start and the end of the study

Test item concentration [mg/L]	pH values	
	0 h	72 h
Control	8.0	8.7
100	7.6	9.7

Comments: The pH value in the test item slightly increased by more than 1.5 pH units. This increase is not regarded to be relevant to the results as all validity criteria were met.

An analysis of the yield and growth rate of the algal population within the 72 h exposure period gave the following results:

Results [mg/L]:

E_rL 50 (0-72 h): > 100

E_rL 10 (0-72 h): > 100

NOEL [r] ($t_{\alpha\ 0.05}$): ≥ 100

LOEL [r] ($t_{\alpha\ 0.05}$): > 100

E_yL 50 (0-72 h): > 100

E_yL 10 (0-72 h): > 100

NOEL [y] ($t_{\alpha\ 0.05}$): ≥ 100

LOEL [y] ($t_{\alpha\ 0.05}$): > 100

No toxic effects against algae were observed at a limit test concentration of 100 mg/L.

PES Vorstufe 2342 is insoluble or poorly soluble in water. Therefore a suitable selective and sensitive chromatographic method for the determination of the test item in aqueous solutions could not be established.

The results are expressed in terms of Effective Loadings (EL). As the test item is a multi constituent and no information about the correlation between molecular weight and the structural formula of the test item are available, a Water Accommodated Fraction (WAF) was used to test effects at a limit concentration of 100 mg/L, and no specific analysis was performed. With the sponsor's agreement, the content of the test item during the exposure period was verified by DOC determination.

The growth curves are presented in **figure 1.**

The nutrient media for pre cultures and test cultures are given in **annex 1**. The equations used to calculate the algal yield and growth rates as well as their percentage inhibition are presented in **annex 2**.

9.1 Comments

The pH value in the test item slightly increased by more than 1.5 pH units. This increase is not regarded to be relevant to the results as all validity criteria were met.

9.2 Validity of the test

According to OECD 201, the factor of the biomass parameter, measured in the control between 0 and 72 h, must be at least 16.

With the current test it was found to be 91.7. The test fulfills this validity criterion.

Evaluation of the section-by-section growth rates:

The mean of the replicate coefficients of variation in the section-by-section growth rate was: 11.3%.

According to OECD 201, the mean coefficient of variation, measured in the control from 0 to 72 h, must not be higher than 35%. The test fulfills this validity criterion.

The coefficient of variation of the mean specific growth rate replicates in the control between 0 and 72 h was: 1.4%.

According to OECD 201, the coefficient of variation of the mean specific growth rate, measured in the control from 0 to 72 h, must not exceed 7%. The test fulfills this validity criterion.

10. ANNEX 1

10.1 Growth medium of pre cultures and test cultures

Nutrient	Concentration in stock solution	Final concentration in the solution of the pre cultures and test cultures
Stock solution 1: macro-nutrients		
NH_4Cl	1.5 g/L	15 mg/L
$MgCl_2$ x 6 H_2O	1.2 g/L	12 mg/L
$CaCl_2$ x 2 H_2O	1.8 g/L	18 mg/L
$MgSO_4$ x 7 H_2O	1.5 g/L	15 mg/L
KH_2PO_4	0.16 g/L	1.6 mg/L
Stock solution 2: Fe-EDTA		
$FeCl_3$ x 6 H_2O	80 mg/L	80 µg/L
Na_2EDTA x 2 H_2O	100 mg/L	100 µg/L
Stock solution 3: trace elements		
H_3BO_3	185 mg/L	185 µg/L
$MnCl_2$ x 4 H_2O	415 mg/L	415 µg/L
$ZnCl_2$	3 mg/L	3 µg/L
$CoCl_2$ x 6 H_2O	1.5 mg/L	1.5 µg/L
$CuCl_2$ x 2 H_2O	0.01 mg/L	0.01 µg/L
Na_2MoO_4 x 2 H_2O	7 mg/L	7 µg/L
Stock solution 4: bicarbonate		
$NaHCO_3$	50 g/L	500 mg/L

Stock solution 1 and 3 were sterilised by autoclaving (120°C, 20 min.), stock solution 2 and 4 were sterilised by membrane filtration (pore size 0.2 µm). All solutions were stored in the dark at 4°C.

To prepare the growth medium 10 mL of stock solution 1 were mixed with 500 mL sterilised millipore water. Then 1 mL each of the stock solutions 2-4 were added and filled up to 1 litre with sterilised millipore water. Afterward the growth medium was bubbled with sterile, filtered air for at least 1 hour.

Water hardness of the final nutrient medium was 1.3 °dH, corresponding to 22.5 mg/L $CaCO_3$.

11. ANNEX 2

11.1 Equations for calculation

Yield was calculated as the biomass at the end of the test minus the starting biomass for each single replicate of controls and treatments. For each test concentration and control a mean value for yield was calculated.
The percentage inhibition of yield for each test concentration was calculated according to

Equation [1]

$$\% I_y = \frac{(Y_c - Y_T)}{Y_c} \times 100$$

Where

$\% I_y$ was the percentage inhibition for yield,

Y_C was the mean value for yield in the control group,

Y_T was the value for yield for the treatment.

The growth rate [r]

For each test concentration and for the control was calculated according to

Equation [2]

$$r = \frac{\ln N_3 - \ln N_0}{t_3}$$

Where

r was the growth rate,

ln was the natural logarithm,

t_3 was the time of the last measurement at the end of the test,

N_o was the nominal initial cell density,

N_3 was the measured cell density at time t_3.

From the mean values of r the percentage inhibition of growth rate for each test concentration was calculated according to

Equation [3]

$$Ir_i = \frac{r_c - r_i}{r_c} \times 100$$

Where

Ir_i was the percentage inhibition (growth rate) for test concentration i,

r_i was the mean growth rate for test concentration i,

r_c was the mean growth rate for the control.